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# Re-evaluation of methaemoglobin reduction as a screening procedure for glucose-6-phosphate dehydrogenase (G6PD)

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**Summary**

Methaemoglobin reduction method is considered to be an old method of qualitative analysis of glucose-6-phosphate dehydrogenase with low specificity and sensitivity and as such, many are sceptical about the reliability of the outcome of the result of the methodology.The aims of this study were to determine the sensitivity and specificity of methaemoglobin reduction method and examine its suitability as a routine method for the screening of glucose-6-phosphate dehydrogenase in this environment. One hundred children were recruited into the study which was conducted over a period of one year. The children (males and females) were aged between 1 day and 15 years. G6PD screening by methaemoglobin reduction method was carried out on all the subjects, while G6PD qualitative assay using G6PD assay kits by Biolabo (France), was carried out in the first 15 subjects who were G6PD deficient by the qualitative screening and the first 15 subjects who were G6PD normal. This was to compare the qualitative with the quantitative assay results and thereby determine the sensitivity and specificity of the methaemoglobin reduction method. The sensitivity and specificity of methaemoglobin reduction test was found to be 93.3 percent each. The results of the qualitative methaemoglobin reduction test for the G6PD deficiency compared well with the quantitative enzymatic assay. With sensitivity and specificity each of 93.3%, the simple qualitative test is dependable and therefore suitable for screening for G6PD.

**Keywords**: *Methaemoglobin, G6PD screening, enzymopathy, P.faciparum*

**Résumé**

La méthode de la réduction de la méthémoglobine est considérée comme une vielle méthode qualitative d'analyse du G6PD avec un faible spécificité et sensitivité. Plusieurs sont sceptiques par rapport à la précision des résultats d'une telle méthode. Le but de cette étude était de déterminer la sensitivité et la spécificité de la méthode de réduction méthémoglobine et d'examiner son acceptabilité comme test de routine pour l'analyse du G6PD dans cet environnement. Cents enfants de moins de 15 ans étaient recrutés dans cette étude pendant un an. le test du G6PD était fait sur tout les échantillons des 30 participants (15 G6PD et 15 sujets sains) et l'analyse qualitative par le kit de Biolabo (France). Les résultats du test quantitatif et qualitatif de sensitivité et de spécificité de cette méthode étaient de 93.3% chacune. Ces résultats du test qualitatif de la réduction de la méthémoglobine pour le déficit en G6PD étaient bien comparables au test quantitatif, dépendant et acceptable pour le dépistage du G6PD.

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## Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common disease-producing enzymopathy in humans. Inherited as an x-linked disorder, G6PD deficiency affects 400 million people worldwide [1,2] The disease is highly protective against *P.falciparum* malaria which probably accounts for its high gene frequency [2,3,4,12].

The highest prevalence is found among persons of African, Asian, or Mediterranean descent. Severity varies significantly between racial groups because of different variants of the enzyme. Severe deficiency variants primarily occur in the Mediterranean population. The enzymatic variants in the African population have more activity and produce a milder form of the disease [5,6].

Methaemoglobin reduction test (MRT), is one of the qualitative screening tests for G6PD deficient hemizygoous males and female heterozygotes. Haemolytic anaemia due to G6PD deficiency can be severe and even, life threatening and consequently screening should be undertaken for all patients suspected with G6PD deficiency or to have received drugs known to precipitate haemolytic crisis in G6PD

deficient patients. Screening may be useful in blood donors since the use of G6PD deficient blood for transfusion is potentially harmful, especially with severe variants of the enzyme [7,8].

Screening procedures and qualitative assays for G6PD have been described, the former depends on either:

(1) the reduction of a dye (brilliant cresyl blue), which can be observed visually,
(2) the reduction of methaemoglobin (Methaemoglobin reduction test, MRT),
(3) the ascorbate-cyanide test in which the browning of haemoglobin is observed when it is not protected from peroxidation, or
(4) the spot test, involving the visual observation of fluorescence of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) formed in the G6PD reaction.

Among the screening procedures for the detection of G6PD deficiency, MRT has been in wider use in various centres in the developing countries with varied reasons among which are the cost effectiveness, availability of the reagents and non-cumbersome nature of the test procedure.

A lot of doubts had been cast on the sensitivity and specificity of the MRT, despite previous reports of an acceptable sensitivity and specificity. Among the various reports of sensitivity and specificity of MRT was that of the scientific group on standardization for the study of G6PD, who found a sensitivity of 80.0% among female heterozygotes who were G6PD deficient [9] and Sanpavat *et al* [10], who reported sensitivity of 85.7% and specificity of 98.1% among neonate and, sensitivity of 60.0% and specificity of 92.1% among the infants.

In view of the doubt and the varied reports of sensitivity and specificity, and associated influence of climatic condition on the sensitivity and specificity of MRT, it became necessary to re-examine the reliability of the MRT in this environment.

## Materials and methods

The study was carried out in the department of haematology, general outpatients department and department of paediatrics, University of Ilorin Teaching Hospital, Nigeria. The subjects for this study included male and female children from 1 day to 15 years old, who had clinical evidence of jaundice. Subjects who were transfused in the previous 6 weeks and premature babies presenting with jaundice were excluded from this study. Clearance was obtained from the ethical committee of the hospital. Informed consent was obtained from the mothers of these children after adequate explanation and education of what the study entails.

### *Sample collection*

One hundred children were recruited into the study conducted over a period of one year. Three (3) millilitres of blood sample was obtained from the ante cubital vein in infants and young children, and superficial vein on the dorsum of the hand in neonates, after adequate antiseptic preparation. This procedure was carried out by the Paediatricians. The specimen was collected into specimen bottle containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant. G6PD screening was carried out within 6 hours after collection. Other investigations done included full blood count (FBC) and reticulocytes count and haemoglobin (Hb) electrophoresis.

G6PD screening, reticulocyte counts and FBC were repeated 6 weeks later for subjects who were G6PD deficient and had high reticulocyte count during the crisis period. The G6PD results considered were those carried out post haemolytic period when subjects were in a stable state condition.

The initial G6PD screening results obtained on first contact served as an additional control for subjects re-screened post crisis period and thus assisted further in categorizing subjects.

### Method

G6PD screening by methaemoglobin reduction method of Brewer [11,12] was first carried out in this study. G6PD quantitative assay using G6PD assay Kits by Biolabo (France) was carried out in the first 15 subjects who were G6PD deficient by the qualitative screening and the first 15 subjects who were G6PD normal.

The sensitivity and specificity of MRT screening method was determined using Biolabo assay G6PD kits as the standard method. FBC was carried out using the sysmex auto analyser model KX 21 while reticulocyte count was carried out by visual method [13]. Hb electrophoresis was carried out only among subjects $\geq$ 6 months old.

*Statistics and analysis*

Data analysis was by the inferential statistical methods employing the chi- square tests and students t-test. The statistical significance of the data was based on p-value < 0.05. All data analysis was done on a computer statistical package with the EPI INFO version 6.0 software.

## Results

Results of sixty-eight (68) males and thirty-two (32) females were analysed. The mean age of the subjects was 4.8(SD5.0) years. Of the thirty (30) subjects who had both methaemoglobin reduction screening test and G6PD assay by kits (Biolabo), twenty (20) were males while ten (10) were females (Table I).

**Table 1:** Status and sex distribution of subjects screened for G6PD by MRT method

| Sex | G6PD Deficient Frequency | Subjects Percent | G6PD Normal Frequency | Subjects Percent |
|---|---|---|---|---|
| Male | 11 | 73.3 | 9 | 60 |
| Female | 4 | 26.7 | 6 | 40 |
| Total | 15 | 100 | 15 | 100 |

The mean Hb for G6PD deficient subjects was 11.4g/dl (SD3.5) and 11.1g/dl (SD3.9) for G6PD normal subjects. The difference was not statistically significant (P = 0.76). (Table 2) The mean reticulocyte count was 2.8% (SD1.9) among G6PD deficient subjects and 2.9% (SD3.9) in G6PD normal subjects. The difference was not statistically significant (P = 0.76) (Table 2).

Eight (8) subjects were age < 6 months old and were excluded from Hb electrophoresis. Of the remaining twenty two subjects (22), eight subjects (8) were HbSS and only two (2) of the eight subjects with HbSS, were G6PD deficient (Table 3).

The mean activity of G6PD in fifteen (15) subjects who were G6PD deficient was 6.74IU/gHb and fifteen (15) subjects who were G6PD normal was 12.48IU/gHb. The difference in mean G6PD assay activity between G6PD deficient and G6PD normal subjects was statistically significant (P < 0.001) (Table 4).

The overall results show a sensitivity of 93.3% and specificity of 93.3%. (Table 5)

**Table 2:** Mean values of Haemoglobin concentration and Reticulocyte count in G6PD deficient and G6PD normal subjects

| Parameters | G6PDDeficient Subjects | G6PD Normal Subjects | P-Values |
|---|---|---|---|
| Hb (g/dl) | 11.4 (SD3.5) | 11.1 (SD3.9) | 0.76 |
| Reticulocyte count(%) | 2.8 (SD1.9) | 2.9 (SD3.9) | 0.76 |

**Table 3:** Hb Electrophoresis among 22 subjects screened for G6PD

| Status | G6PD Deficient Subjects No (%) | G6PDNormal Subjects No (%) | Total No |
|---|---|---|---|
| AA | 5 (50) | 2 (16.7%) | 7 |
| AS | 3 (30%) | 4 (33.3%) | 7 |
| SS | 2 (20%) | 6 (50%) | 8 |
| SC | - | - | - |
| Total | 10 (100%) | 12 (100%) | 22 |

**Table 4:** Mean G6PD assay results in thirty (30) subjects screened for G6PD deficiency

| Screening status | Mean G6PD assay activity IU/gHb |
|---|---|
| G6PD Deficient | 6.74(SD6.79) (N = 15) |
| G6PD Normal | 12.48(SD4.21)(N = 15) |
| P-Value | < 0.001 |

**Table 5:** Sensitivity and specificity of the methaemoglobin reduction method

| Standard test | G6PD deficient by MRT (Procedure being assessed) | G6PD normal by MRT (Procedure being assessed) | Total |
|---|---|---|---|
| G6PD Deficient by Kit | 14 | 1 | 15 |
| G6PD Normal by Kit | 1 | 14 | 15 |
| Total | 15 | 15 | 30 |

*Sensitivity* = 14/15 = 93.3%
*Specificity*= 14/15 = 93.3%

## Discussion

The concept of G6PD screening by methaemoglobin reduction method was conceived by Brewer *et al* in 1962 and had been in use since then [11]. There has

been various doubt and misconception about the sensitivity of MRT in the detection of G6PD deficient patients [15]. This methodology offers special advantages, especially to the health care giver in the developing countries and poor communities like ours in view of its cost effectiveness, being less cumbersome, less reliant on sophisticated facility, and less electricity dependent, when compared to quantitative methods. The number of samples used for the calculation of sensitivity and specificity was limited to thirty (30) due to the cost of reagent.

The World Health Organization in a report published in 1966, titled standardization of procedures for the study of glucose-6-phosphate dehydrogenase, found MRT to be 80.0% sensitive for female heterozygotes who were G6PD deficient [9]. In this study, MRT was found to be 93.3% using the G6PD assay kits as the standard. This finding is similar to earlier report of Sanpavat *et al*, in 2001 who demonstrated an acceptable sensitivity of 85.7% and high specificity of 98.1% among infants [10].

However, our finding differs from Sanpavat *et al*. subsequent reports of the finding of low sensitivity of 60.0% among jaundiced neonate [10]. Low sensitivity is most likely when the test is carried out during the period of an acute haemolytic crisis because of the preponderance in the circulation, of young erythrocytes (reticulocytes) which are rich in G6PD. In this study, tests were carried out post haemolytic period, when the subjects were in a stable state condition. This was reflected in the mean Hb of 11.4g/dl (SD3.5) for the G6PD deficient subjects and 11.1g/dl (SD3.9) for G6PD normal subjects; and in the mean reticulocyte count of 2.8% (SD1.9) for G6PD deficient and 2.9% (SD3.9) for G6PD normal subjects.

The value of MRT had been described in several studies and a comparable result had been obtained relative to other methods of G6PD determination. One of such results can be found in the study by Kuptamethi *et al*, 1992 who reported that MRT was equally as efficient as spectrophotometrics and cytometric methods in the detection of hemizygous G6PD deficiency in HbH patients although similar result was not obtained in G6PD deficient females [14].

MRT, although considered to be less sensitive in some reports [15], can still be said to be a reliable alternative especially where resources are limited as obtained in the developing countries. While the cost per detection by MRT amounted to about N300.00, the cost per detection of G6PD activity by kits is N2,500.00 in most centres.

In this study, the results of the qualitative reduction test for the detection of G6PD deficiency correlate well with the quantitative enzymatic assay. With sensitivity and specificity of 93.0% each, this simple qualitative test although old, remains reliable and suitable for screening for G6PD deficiency in our environment.

Finally, it is important to note that MRT screening gives the best results if done in the post haemolytic period when high reticulocyte counts of the crisis period have normalized and so it should be used in this condition.

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